

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



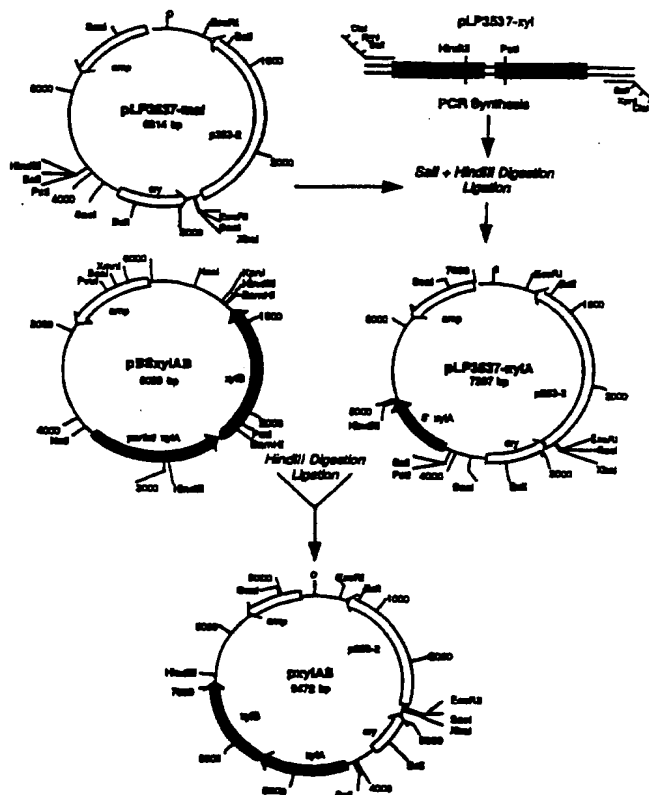
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 1/20, 15/74, C12P 7/56	A1	(11) International Publication Number: WO 97/13842 (43) International Publication Date: 17 April 1997 (17.04.97)
(21) International Application Number: PCT/US96/15442 (22) International Filing Date: 27 September 1996 (27.09.96) (30) Priority Data: 08/541,632 10 October 1995 (10.10.95) US (71) Applicant: MIDWEST RESEARCH INSTITUTE [US/US]; 425 Volker Boulevard, Kansas City, MO 64110 (US). (72) Inventors: PICATAGGIO, Stephen, K.; 23870 Genesee Village Road, Golden, CO 80401 (US). ZHANG, Min; 14178 W. Warren Drive, Lakewood, CO 80228 (US). FRANDEN, Mary, Ann; 3653 E. Lake Drive, Littleton, CO 80121 (US). MC MILLAN, James, D.; 1277 Aikins Way, Boulder, CO 80303 (US). FINKELSTEIN, Mark; 4321 Whippeny Drive, Fort Collins, CO 80526 (US). (74) Agent: RICHARDSON, Ken; National Renewable Energy Laboratory, Assistant Chief Patent Counsel, 1617 Cole Boulevard, Golden, CO 80401 (US).	(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published With international search report. With amended claims.	

(54) Title: RECOMBINANT LACTOBACILLUS FOR FERMENTATION OF XYLOSE TO LACTIC ACID AND LACTATE

(57) Abstract

A recombinant *Lactobacillus* MONT4 is provided which has been genetically engineered with xylose isomerase and xylulokinase genes from *Lactobacillus pentosus* to impart to the *Lactobacillus* MONT4 the ability to ferment lignocellulosic biomass containing xylose to lactic acid.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

Recombinant Lactobacillus for Fermentation of
Xylose to Lactic Acid and Lactate

5 Contractual Origin of the Invention

The United States Government has rights in this invention under Contract No. DE-AC36-83CH10093 between the United States Department of Energy and the National Renewable Energy Laboratory, a Division of Midwest Research Institute.

10

Technical Field

This invention relates to a microorganism which has been genetically altered to permit fermentation of xylose to lactic acid and lactate.

15 Background Art

Lactic acid, and its salt known as lactate, are commercially viable products useful in various fields including medicine and food processing. Currently, lactic acid is commercially produced from cornstarch or sucrose. Lignocellulosic biomass offers a favorable alternative as a feedstock for the biological production of lactic acid because it is readily available, has no competing food value, and is less expensive than either cornstarch or sucrose. Theoretically, a microorganism would be able to ferment the sugars contained in the biomass to lactic acid. However, several obstacles preclude efficient utilization of this feedstock by a microorganism for lactic acid production. Lignocellulosic feedstocks are largely composed of cellulose, hemicellulose and lignin. While several micro-organisms can efficiently ferment the glucose component in cellulose, conversion of the pentose sugars contained in the hemicellulose fraction has proven more difficult. The most abundant pentose sugars in hemicellulose include xylose and arabinose. Fermentation of xylose and arabinose remains a primary obstacle for economical biomass conversion. The few microorganisms that can grow on both pentose and hexose sugars in lignocellulosic feedstocks typically grow slowly and demonstrate marginal yields and productivities. Because the cost of feedstock can represent more than 20% of all process costs, an economical biomass conversion process critically depends upon the rapid and efficient conversion of essentially all of the sugars present in both the cellulose and hemicellulose fractions. Therefore, an expanded substrate utilization range

which includes efficient conversion of both hexose and pentose sugars is an important requirement of such a microorganism.

In addition to an expanded substrate utilization range, such a microorganism will be required to tolerate and perform at varying process conditions including elevated temperature, low pH, and high salt concentration. Previous studies have shown that most microorganisms demonstrate relatively poor pentose sugar fermentation performance on dilute-acid hydrolyzates compared to laboratory formulations containing pure sugars (McMillan, J., *Conversion of Hemicellulose Hydrolyzates to Ethanol*, Chapter 21, Enzymatic Conversion of Biomass for Fuels Production, in ACS Symposium Series 566, pp. 411-437, 1994). The lower conversion yield and productivity from dilute-acid hydrolyzates have been attributed to the presence of inhibitory compounds such as acetic acid, furfurals and assorted phenolics. Therefore, in addition to fermenting under ranging process conditions the variety of sugars found in lignocellulosic biomass, the microorganism must also be able to efficiently ferment biomass-derived sugars in the presence of these inhibitory components. The present invention provides a microorganism with these desirable characteristics and abilities.

Lactobacillus are used commercially in the preparation of a variety of food and feed products (Chassy, *Trends in Biotechnol.*, 3, pp. 273-275, 1985) providing several potential advantages for biomass conversion. *Lactobacillus* are gram-positive, non-spore-forming bacteria capable of fermenting many of the carbohydrates commonly found in lignocellulosic biomass, such as glucose, starch, cellobiose, lactose, xylose, arabinose and ribose, and produce high concentrations of lactate (Kandler, O., *Antonie van Leeuwenhoek*, 49, pp. 209-244, 1983; Gold et al., *J. Industrial Microbiol.*, 10, pp. 45-54, 1992). The fermentations may be conducted at low pH and elevated temperatures, thus allowing the use of process conditions which minimize contamination. *Lactobacillus* show considerable resistance to the inhibitory compounds found in dilute-acid hydrolyzates (Olsson et al., *Appl. Biochem. Biotechnol.* 34/35, pp. 359-368, 1992). In addition, many *Lactobacillus* are Generally Recognized As Safe (GRAS) for use at industrial scale.

Pentose sugars, such as xylose and arabinose, are readily fermented by facultative heterofermentative strains to equimolar amounts of lactate and acetate via the phosphoketolase pathway. However, glucose and xylose consumption rates are relatively low compared to other fermentative microorganisms (Olsson et al., *supra*). In contrast,

obligate homofermentative strains of *Lactobacillus* can ferment glucose to lactate as the sole fermentation product at over 95% of the maximum theoretical conversion yield (Gold et al., supra). Catabolism occurs via glycolysis, converting 1 mol of hexose to 2 mol of lactic acid. Unfortunately, these strains are incapable of fermenting pentose sugars. A survey of 31 different *Lactobacillus* strains identified several that were capable of at least 90% conversion of glucose, cellobiose, lactose or starch to a mixture of lactic acid, ethanol and acetic acid (Gold et al., supra). No strains were found that were able to convert xylose at similar efficiency.

Lactobacillus MONT4, originally isolated from high-temperature fermenting grape musts (Barre, P., *Journal of Applied Bacteriology*, 44, pp. 125-129, 1978), is unique in that it ferments L-arabinose and D-ribose exclusively to lactic acid by a homofermentative metabolic pathway unknown among other lactobacilli, which typically ferment pentose sugars via the heterofermentative phosphoketolase pathway. *Lactobacillus* MONT4 is further distinguished from other homofermentative lactobacilli, such as *L. amylovorous*, *L. delbrueckii*, *L. farciminis*, *L. helveticus* and *L. salivarius*, by its unique sugar utilization pattern. Whereas *Lactobacillus* MONT4 can ferment arabinose, it is incapable of fermenting the xylose commonly found in lignocellulosic feedstocks. Thus far, the xylose operon from *Lactobacillus pentosus* has been cloned and expressed only in other heterofermentative lactobacilli (Posno et al., *Appl. Environ. Microbiol.* 57, pp. 2764-2766, 1991). Attempts to transform thermophilic lactobacilli have been largely unsuccessful (Thompson, K. and Collins, M., *Appl. Microbiol. Biotechnol.*, 35, pp.334-338, 1991).

All references cited in this patent application are incorporated herein by reference.

Disclosure of the Invention

It is an object of the present invention to provide a homofermentative strain of *Lactobacillus* MONT4 capable of fermenting pentose sugars including xylose and arabinose at near theoretical yield. This is an ideal microorganism for economical lactate production from renewable biomass substrates.

It is a further object of the present invention to provide a method for producing lactic acid from hemicellulose hydrolyzates of lignocellulosic biomass.

This invention relates to a *Lactobacillus* MONT4 strain that has been metabolically engineered for xylose fermentation by introducing the xylose isomerase and

5 xylulokinase genes that link xylose assimilation to its homofermentative glycolytic pathway. This engineered strain produces lactate as the sole fermentation product from xylose at near-theoretical yield (about 1.67 moles lactate/mole xylose) and has potential commercial application for lactic acid and lactate production from lignocellulosic feedstocks because it also ferments many of the other sugars commonly found in these feedstocks, including glucose, cellobiose, mannose and arabinose.

Brief Description of the Drawings

10 Figure 1 sets forth data comparing the lactic acid fermentation performance of transformed and untransformed MONT 4 on xylose and xylose plus glucose.

Figure 2 is a series of plasmid maps showing the generation of pxy/AB.

Description of the Preferred Embodiments

15 The invention is a recombinant *Lactobacillus* with an expanded substrate utilization range which is capable of growth on and/or efficient lactic acid production from xylose or other pentoses, as well as hexoses, as its sole carbon source.

20 The microorganisms used to prepare the present invention are those which are capable of being genetically altered to produce the necessary enzymes to form a metabolic pathway for catabolizing pentoses, particularly xylose. The microorganism may naturally have some enzymes in the pathway but will not be able to ferment xylose into lactic acid until it has been genetically altered. The manner of genetic alteration may use any combination of known genetic engineering techniques such as mutation or addition of foreign DNA, provided that the microorganism is able to ferment xylose to lactic acid after treatment. Foreign DNA may be introduced into the microorganism by any
25 conventional technique such as conjugation, transformation, transduction or electroporation.

30 Many microorganisms which are capable of fermenting sugars to lactic acid lack at least one of the genes for the enzymes which make up a metabolic pathway for converting xylose and other pentoses into lactic acid. Exogenous genes may be added to complete a metabolic pathway. One need not add genes necessary for every step if the host micro-organism already produces an enzyme in the pathway. The number of genes to be added will depend on the starting microorganism.

The indigenous *Lactobacillus* genes may be altered by any known genetic manipulation technique to provide a protein with the necessary enzyme activity to produce the desired metabolic pathway. The altered genes may complement one or more of the introduced genes from another organism to complete the metabolic pathway. The use of this procedure may be advantageous by reducing the number of genes one needs to add to the host cell.

Sufficient genes may be added so that the recipient microorganism may ferment xylose as the sole carbon source. The microorganism may or may not be able to multiply on xylose as the sole carbon source but may be capable of fermenting xylose to lactic acid.

A gene may be added to a cell by way of a vector. The vector may be in the form of a plasmid, cosmid or virus which is compatible with the cell's DNA and any resident plasmids. Generally, vectors either integrate into the recipient microorganism's DNA or the vector has an origin of replication to stably maintain the vector throughout many microbial generations. The origin of replication may code for either stringent or non-stringent replication.

To express the gene(s), a structural gene is generally placed downstream from a promotor region on the DNA. The promotor must be recognized by the recipient microorganism. In addition to the promotor, one may include, delete or modify regulatory sequences to either increase expression or to control expression. Expression may be controlled by an inducer or a repressor so that the recipient microorganism expresses the gene(s) only when desired.

In a preferred embodiment of the invention, xylose metabolic pathway genes are obtained from xylose metabolizing microorganisms and added to microorganisms which do not otherwise ferment xylose to lactic acid. As an example, the xylose metabolizing genes from *Lactobacillus pentosus* are added to *Lactobacillus* MONT4.

In other preferred embodiments of the present invention, a genetic element comprising two genes enabling xylose metabolism may be placed on the same vector. The genes on a vector may be in any order, grouping or orientation relative to each other. The expression of the genes and the resulting functional activity of their corresponding gene products represent a new biochemical pathway that links xylose metabolism to the Pentose Phosphate and Embden-Meyerhof Parnas pathways in *Lactobacillus*, conferring upon these cells, for the first time, the ability to grow on and ferment xylose directly to lactate.

The microorganism according to the present invention may be mixed with any xylose, arabinose or other pentose sugar containing medium and allowed to ferment the medium to produce lactic acid. The medium may include other fermentable sugars, such as glucose. If microbial growth is desired, other nutrients which are necessary for microbial growth may be added and the microorganism allowed to reproduce.

Efficient transport of the pentoses into *Lactobacillus* may be through native *Lactobacillus* transport proteins, mutated *Lactobacillus* transport proteins, or through the addition of new facilitated transporters introduced by cloning new transport genes into *Lactobacillus* with or without mutagenesis of the cloned transport genes.

The step of microbial growth may be separate from fermentation. Xylose, arabinose and other pentoses, or mixtures thereof may be used as a carbon source for microbial growth or one can separately culture the microorganism on any medium (with or without a pentose) until sufficient numbers of microorganisms are present as a first step and then a pentose containing medium is added for fermentation in a second step. If a two step method is used, one may control expression of the genes in the new metabolic pathway so that greater expression occurs during the second step.

The choice of substrates will depend on cost and supply of the substrate to be fermented to lactic acid. A typical low-cost supply of pentoses is from hemicellulose. Xylose, arabinose and other pentoses are liberated from hemicellulosic materials by treatment with steam and/or an acid or alkali. Smaller amounts of other sugars such as glucose are also separated during this treatment and are also fermented by *Lactobacillus* to lactic acid.

When the substrate is cellulosic materials, the cellulose may be hydrolyzed to sugars simultaneously or separately and also fermented to lactic acid. Since hemicellulose is generally easier to hydrolyze to sugars than cellulose, it is preferable to first prehydrolyze the hemi-cellulosic material, separate the soluble pentose sugars and then hydrolyze the cellulose by treatment with steam and/or acid or alkali or cellulases to form glucose. Both pentose and hexose sugars may be simultaneously or separately fermented to lactic acid using the microorganism of the present invention. If so desired, the hexoses may be fermented by a different microorganism to lactic acid, such as yeast, natural *Lactobacillus*, etc.

Many fermentation conditions are known per se as shown by the references mentioned in the Background of the Invention section above. Accordingly, the range of

fermentation conditions may be quite broad. Likewise, any of the many known types of apparatus may be used for the present invention.

5 The microorganism according to the present invention may be used as a biologically pure culture or it may be used with other lactic acid producing microorganisms in mixed culture. Biologically pure cultures are generally easier to optimize but mixed cultures may be able to utilize additional substrates. One may also add enzyme(s) to the fermenter to aid in the degradation of substrates or to enhance lactic acid production. For example, cellulase may be added to degrade cellulose to glucose simultaneously with the fermentation of glucose to lactic acid by microorganisms. 10 Likewise, a hemicellulase may be added to degrade hemicellulose.

In the preferred embodiment using a genetically engineered *Lactobacillus*, fermentation broth cultures are relatively resistant to contamination by other microorganisms. Nonetheless, it is preferred to eliminate or disable preexisting deleterious microorganisms in the substrate added to the *Lactobacillus* culture.

15 After fermentation, the lactic acid or lactate is separated from the fermentation broth by any of the many conventional techniques known to separate lactic acid or lactate from aqueous solutions. Particles of substrate or microorganisms may be removed before separation to enhance separation efficiency.

20 While the discussion of the fermentation in this specification generally refers to a batch process, parts or all of the entire process may be performed continuously. To retain the microorganisms in the fermenter, one may separate solid particles from the fluids. Alternatively, the microorganisms may be immobilized for retention in the fermenter or to provide easier separation.

25 Unless specifically defined otherwise, all technical or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are better illustrated by the use of the following non-limiting examples. The following examples are offered by way of illustration and not by 30 way of limitation.

Example 1

Demonstration of a Unique Inducible Pentose
Phosphate Pathway in *Lactobacillus* MONT4

5 *Lactobacillus* MONT4, originally isolated from high-temperature fermenting grape
musts (Barre, P. supra) was obtained from the German culture collection DSM
(Mascheroder Weger 1B - 33000 Braunschweig, Germany) under the designation DSM
20605. This microorganism is unique in that it ferments L-arabinose and D-ribose
exclusively to lactic acid, apparently by a metabolic pathway unknown among other
10 lactobacilli, which typically use the phosphoketolase pathway. *Lactobacillus* MONT4
was cultured at 37°C for 18 hours in MRS broth (pH 6.7), containing 10 g/l proteose
peptone no. 3 (Difco Laboratories); 10 g/l beef extract (Difco); 5 g/l yeast extract (Difco
Laboratories); 1 g/l Tween 80 (Difco); 5 g/l sodium acetate·3H₂O (Sigma); 2 g/l dibasic
potassium phosphate (Sigma); 2 g/l triammonium citrate (Sigma); 0.2 g/l magnesium
15 sulfate·7H₂O (Sigma); 0.05 g/l manganese sulfate·4H₂O (Sigma); and either 20 g/l D-
glucose (Sigma), D-xylose (Sigma) or L-arabinose (Sigma). Growth was monitored by
measuring absorbance at 600nm using a Beckman DU640 spectrophotometer. The cells
were collected by centrifugation, washed in 50mM Tris-HCl pH 7.6 containing 1mM DTT
(TD), and resuspended in TD. Cell pellets were frozen at -80°C until ready to use. Cell-
20 free extracts were prepared and analyzed for the presence of xylose assimilation (xylose
isomerase and xylulokinase) and pentose phosphate pathway enzymes (transaldolase and
transketolase) as follows: the cells were thawed and ruptured using Aminco French
Pressure Cell Press operated at 1000 psi gauge pressure or 20,000 psi cell pressure. Cell
debris was removed by centrifugation (17,000xg, 30 min, 4°C). Protein determinations
25 were performed using the Bio-Rad Protein Assay, based on the Bradford dye-binding
procedure (Bradford, M., *Anal. Biochem.* 72, p248, 1976), using BSA as the standard.

 Xylose isomerase activity was measured by a modification of the cysteine-
carbazole method (Schlief, R.F. and Wensink, P.C., *Practical Methods in Molecular*
Biology, Enzyme Assays, pp. 46-50) in a reaction mixture containing 50mM Tris-HCl (pH
30 7.6), 100mM D-xylose and 20mM MnCl₂. Cell-free extracts were diluted in 50mM Tris-
HCl (pH 7.6), added to an equal volume of reaction mix (100μl total volume), and
incubated for 10 minutes at 37°C before the reaction was terminated by the addition of
900μl of 0.1N HCl. 100μl of freshly prepared 1.5%(w/v) cysteine-HCl, 100μl of
0.12%(w/v)carbazole in 95% ethanol, and 3 ml of 70% sulfuric acid were added to the

reaction mixture and the absorbance at 550nm was measured after a 30 minute incubation at room temperature. The absorbance was compared to a linear standard curve prepared by using D-xylulose in the reaction mix. Units are measured as μ moles xylulose formed per minute per mg of protein. Xylulokinase activity was measured as described by Feldmann et. al., (*Mol. Gen. Genet.* 234, pp. 201-210, 1992).

Transaldolase and transketolase activities were measured by a modification of the methods described by Menezes et. al., (*Indian Journal of Biochemistry & Biophysics* 27, pp.18-22, 1990) and Feldmann et al., supra. The reaction mixture for the transketolase assay contained 50mM Tris-HCl (pH 7.6), 10mM $MgCl_2$, 1.1mM erythrose-4-phosphate (Sigma), 1.1mM co-carboxylase (TPP-Sigma), 0.2mM xylulose-5-phosphate (Sigma), 3.6 units of phosphoglucose isomerase (Boehringer Mannheim), 0.2 units of glucose-6-phosphate dehydrogenase (Boehringer Mannheim), and 0.37 mg of NADP (Sigma) in a 500 μ l volume. The reaction mixture for the transaldolase assay contained 50mM Tris-HCl (pH 7.6), 20mM EDTA, 1.1mM D-sedoheptulose-7-phosphate (Sigma), 1.1mM D,L-glyceraldehyde-3-phosphate from diethylacetal, monobarium salt (Sigma), 3.6 units of phosphoglucose isomerase, 0.2 units of glucose-6-phosphate dehydrogenase, and 0.37 mg of NADP (Sigma) in a 500 μ l volume. The formation of NADPH was monitored by absorbance at 365nm over time. One unit of enzyme activity is defined as 1 μ mole NADPH/minute/mg protein.

Xylose assimilation and pentose phosphate pathway enzyme activities were determined for *Lactobacillus* MONT4, grown in MRS media supplemented with either 2% glucose or 2% arabinose; *L. casei rhamnosus* (NRRL-B445), previously designated as *L. delbrueckii delbrueckii*, grown in MRS media supplemented with 2% glucose; and *L. brevis* (IFO 3960), grown in MRS media supplemented with either 2% glucose or 2% xylose. The facultative homofermentative *L. casei rhamnosus* (NRRL-B445) does not utilize xylose or contain a pentose phosphate pathway but metabolizes ribose via an inducible phosphoketolase pathway. In contrast, the obligate heterofermentative *L. brevis* (IFO 3960) metabolizes both hexose and pentose sugars, including xylose, via the phosphoketolase pathway and served as a positive control for measurement of both xylose isomerase and xylulokinase activities.

Table 1

Enzymatic Activities of *Lactobacillus* (μ moles/min-mg)

Sample	Substrate	Xylose Isomerase	Xylulose Kinase	Trans-ketolase	Trans-aldolase
NRRL-B445	glucose	<0	0.002	0.004	0.003
MONT4	glucose	<0	0.011	0.013	0.018
MONT4	arabinose	<0	0.006	0.200	0.172
IFO 3960	glucose	<0	0.006	<0	0.031
IFO 3960	xylose	1.099	0.028	<0	0.016

The results shown in Table 1 demonstrate the presence of the transaldolase and transketolase activities characteristic of the non-oxidative portion of the pentose phosphate pathway in *Lactobacillus* MONT4 grown in the presence of arabinose. No significant transaldolase or transketolase activities were detected in either *Lactobacillus* MONT4 or *L. casei rhamnosus* grown in the presence of glucose, or in *L. brevis* grown in the presence of either glucose or xylose. These results demonstrate the presence of a unique inducible pentose phosphate pathway in *Lactobacillus* MONT4, previously unknown, which distinguishes *Lactobacillus* MONT4 from other lactobacilli in its ability to ferment a pentose sugar, such as arabinose, via an inducible pentose phosphate pathway. The results also indicate the absence of the xylose isomerase and xylulokinase activities necessary for xylose assimilation in *Lactobacillus* MONT4.

Example 2

Development of a Xylose-Fermenting Strain of *Lactobacillus* MONT4

To develop a xylose-fermenting strain of *Lactobacillus* MONT4 suitable for fermentation of the xylose component in lignocellulosic feedstocks, *Lactobacillus* MONT4 was transformed with plasmid pLP3537-*xyl* (Posno et al., supra), containing the *L. pentosus* xylose operon by a modification of the procedure described by Posno et al. (*Appl. Environ. Microbiol.* 57, pp. 1822-1828, 1991). This modification is described in Example 3, below. To prepare competent cells, a log-phase culture of *Lactobacillus*

MONT4 was diluted 1:1000 and grown overnight in MRS media (Difco) containing 2% (w/v) glucose. The cells were harvested by centrifugation at 7000 rpm for 15 minutes at 15°C using a Sorval GSA rotor, washed twice with an equal volume of cold, sterile water and resuspended in 3 ml of sterile, cold 30% (v/v) PEG 1450 (Sigma). The cells were collected by centrifugation at 17,000 x g for 2 minutes and resuspended in 30% (v/v) PEG 1450 to an approximate OD₆₀₀ of 45. For electroporation, 0.1 µg pLP3537-xyl in 5 µl of TE buffer was added to 50µl of competent cells and the cells were electroporated in 0.2 cm cuvettes at 25 µF, 12.5 KV/cm, 200Ω using a BioRad Gene Pulser.

After electroporation, cells were incubated for 3-4 hours at 37°C in MRS media containing 2% (w/v) glucose. Cell suspensions were then plated onto MRS agar (Difco) plates containing 2% (w/v) glucose and 10µg/ml erythromycin and incubated at 37°C for up to 5 days. Plasmid DNA from erythromycin-resistant transformants was isolated according to the procedure described by O'Sullivan and Klaenhammer, (*Applied and Environmental Microbiology*, 59, pp.2730-2733, 1993) using both mutanolysin (Sigma) and lysozyme (Sigma) to aid in cell lysis. Restriction analysis of the plasmid DNA isolated from erythromycin-resistant transformants confirmed the presence of pLP3537-xyl in *Lactobacillus* MONT4. Further analysis of the erythromycin-resistant transformants using API 50 CH strip tests (bioMérieux) also showed their sugar utilization patterns to be typical of *Lactobacillus* MONT4 prior to transformation with pLP3537-xyl, with the exception that transformants harboring pLP3537-xyl were also capable of growth on MRS agar plates containing 20g/l D-xylose, 20µg/ml erythromycin and 0.017% bromocresol purple. Transformants containing plasmid pLP3537-xyl demonstrated growth on xylose as the sole-carbon source and the formation of yellow halos surrounding colonies. This is the first example of genetic transformation of *Lactobacillus* MONT4 with heterologous DNA to create a xylose-fermenting strain of *Lactobacillus* MONT4.

To test the fermentation performance of *Lactobacillus* MONT4 (pLP3537-xyl), cells were inoculated into MRS broth containing either 20 g/l xylose, 20 g/l glucose or 10 g/l of both glucose and xylose and were incubated at 37°C. Growth was monitored by measuring the OD₆₀₀ using a Spectronic 601 spectrophotometer. Samples were taken at selected time intervals for measurement of sugar utilization and product formation. The presence of organic acids was determined by using a Hewlett Packard LC1090 HPLC equipped with an HP1047A RI detector and a Bio-Rad Aminex 300x7.8mm HPX-87H

ion exclusion column heated to 65°C. The mobile phase was .01N H₂SO₄ run at 0.6 ml/min.

The results shown in Figure 1 demonstrate the ability of *Lactobacillus* MONT4 (pLP3537-xyl) to ferment xylose to lactic acid at 94% of the maximum theoretical yield, based on the amount of consumed sugar. This is the first demonstration of a *Lactobacillus* with the ability to exclusively ferment xylose to lactic acid via the pentose phosphate pathway. The results also showed that only approximately 65% of the available xylose was consumed. In addition, xylose utilization was completely repressed in the presence of 1% (w/v) glucose. In contrast to the results previously reported for *Lactobacillus* MONT4 (Barre, P., supra), both *Lactobacillus* MONT4 and *Lactobacillus* MONT4 (pLP3537-xyl) consumed only approximately 65% of the available arabinose in MRS media containing 20 g/l L-arabinose after 96 hours at 37°C. Analysis of individual colonies from the original *Lactobacillus* MONT4 culture revealed two different cell types. Analysis of both types using API 50 CH strip tests revealed identical sugar utilization patterns. However, one type consistently consumed only approximately 65% of the available arabinose and at a slower rate compared to the other type. The strain demonstrating faster and complete arabinose consumption was designated as *Lactobacillus* MONT4⁺ and was subsequently transformed with plasmid pLP3537-xyl as described above.

The fermentation performance of *Lactobacillus* MONT4⁺ (pLP3537-xyl) was determined in MRS media containing 10 µg/ml erythromycin and either 20 g/l D-glucose, 20 g/l D-xylose, 20 g/l L-arabinose, 10 g/l of both D-glucose and L-arabinose, 10 g/l of both D-glucose and D-xylose, or 10g/l of both D-xylose and L-arabinose. Inocula were passaged twice in MRS media containing 10 µg/ml erythromycin and 20 g/l glucose, 20 g/l xylose or 20 g/l arabinose prior to inoculation of mid-log phase cells into fermentation media to an initial OD₆₀₀ of 0.08-0.1. Control fermentations were run with wild-type *Lactobacillus* MONT4⁺ for comparison to *Lactobacillus* MONT4⁺ (pLP3537-xyl), with the exception that erythromycin was not added to the controls. The cultures were incubated at 37°C and growth was monitored by measuring the OD₆₀₀ over time using a Spectronic 601 spectrophotometer. Samples were taken at selected time intervals for measurement of sugar utilization and product formation. The conversion yields based on either sugar consumed in 143 hrs (Yp/s) or total available sugars (Yp) are summarized in Table 2.

Table 2
Conversion Yields with *Lactobacillus* MONT4⁺ (pLP3537-xyl)

Fermentation Substrate	Inocula Substrate	Yp	Yp/s
2% Glucose	Glucose	92%	92%
2% Xylose	Xylose	86%	98%
2% Arabinose	Arabinose	96%	96%
1% Xyl+ 1% Ara	Xylose	82%	97%
1% Xyl+ 1% Ara	Arabinose	84%	99%
1% Xyl+ 1% Glu	Xylose	49%	93%

The results presented in Table 2 show that the theoretical lactate yields based on the amount of consumed sugar (Yp/s) were greater than 92% for all sugars and mixtures of sugars tested. When grown in the presence of 2% xylose, *Lactobacillus* MONT4+ (pLP3537-xyl) utilized 86% of the total available xylose after 120 hours of incubation, an improvement over the approximately 65% lactate yields previously observed with *Lactobacillus* MONT4 (pLP3537-xyl). The presence of 1% glucose completely repressed xylose metabolism. When *Lactobacillus* MONT4+ (pLP3537-xyl) was grown in the presence of 1% xylose and 1% arabinose, all the available arabinose and approximately 65% of the available xylose was utilized after 24 hours and 144 hours, respectively.

Example 3

Reduction of Glucose Catabolite Repression of Xylose Utilization

For an efficient industrial process, it is necessary to alleviate glucose catabolite repression of pentose assimilation. The expression of the wild-type *L. pentosus* MD353 *xylAB* operon is negatively regulated at the level of transcription by a repressor, the product of *xylR*, and transcribed from a different promoter (Lokman et. al., *Mol. Gen. Genet.* 245, pp.117-125, 1994). Thus, elimination of the *xylR* gene upstream of *xylA* might reduce glucose catabolite repression of the *xylAB* genes.

Therefore, a cloning strategy was designed to isolate the xylose isomerase (*xylA*) and xylulokinase (*xylB*) genes from the xylose operon contained on pLP3537-xyl by PCR synthesis followed by subcloning into the shuttle vector, pLP3537 (Leer et. al., *Mol. Gen. Genet.*, 234, pp.265-274, 1992). By eliminating the gene for *xylR*, regulation of *xylA* and

xylB transcription might be reduced or even eliminated. To this end, two oligonucleotides were constructed (Macromolecular Sequences) for gene amplification via PCR:

5'-Primer #1: CCA TCG ATG GTA CCG TCG ACG TTC TAG AAA GCG **TTTAC**
(SEQ ID NO: 1)

3'-Primer #2: CCA TCG ATG GTA CCG TCG ACA AGA CAC GTA AAA AAT CGC
(SEQ ID NO: 2)

The first 20 bases of each oligonucleotide were designed to include three restriction sites for *ClaI*, *KpnI* and *SalI* to facilitate subsequent subcloning. The sequence in bold letters of oligonucleotide #1 is complimentary to the 5'-DNA sequence upstream of the xylose isomerase gene (*xyIA*). The DNA sequence for the *L. pentosus* xylose operon can be found in GenBank under the accession number M57384. The underlined sequence includes the -35 region of the promoter for *xyLAB*. The sequence in bold letters for oligonucleotide #2 is the inverse complement of the sequence downstream of *xyIB* and includes a potential transcription terminator. The PCR was performed with oligos #1 and #2 at .2mM dNTP (Perkin Elmer) in a reaction volume of 0.1 ml containing 50 pg pLP3537-*xyl*, 0.2 mg/ml BSA (New England Biolabs), 2 units Vent_r DNA Polymerase and buffer supplied by the manufacturer (New England Biolabs). Temperature cycling conditions were 1 min at 94°C, 2 min at 50°C and 3 min at 72°C for 25 cycles. The expected 3.2 kb DNA fragment, designated *xyLAB*, was isolated by preparative agarose gel electrophoresis using GeneClean (BIO 101). However, repeated attempts to subclone *xyLAB* into *E. coli* XL-1 Blue using a pCR-ScriptTMSK(+) (Stratagene) as described by the manufacturer were unsuccessful. A single transformant was obtained, however DNA sequence analysis showed that 400 bases at the 5' end of the PCR product were deleted. The recovered plasmid, designated pBS*xyLAB*, contained the 3' end of *xyIA* and the entire *xyIB* gene. Although the parent plasmid pLP3537-*xyl* could be successfully transformed into *E. coli* XL-1 Blue, the subcloning of *xyLAB* into pCR-ScriptTMSK(+) apparently resulted in a structurally unstable plasmid in *E. coli*.

Therefore, a two-step cloning strategy was designed to first introduce the 5' end of *xyIA* followed by the rest of the *xyLAB* structural genes (Figure 2). A modified plasmid was first constructed by removing the xylose operon from pLP3537-*xyl* by digestion with *PstI* and *HindIII*, and then religating the plasmid with two annealed oligonucleotides

*Pst*I and *Hind*III, and then religating the plasmid with two annealed oligonucleotides having *Pst*I and *Hind*III overhangs and internal *Sa*I and *Bg*III restriction sites. The sequence for both oligonucleotides follows:

5

5' GGTCGACAGATCTA 3' (SEQ ID NO: 3)

5' AGCTTAGATCTGTCGACCTGCA 3' (SEQ ID NO: 4)

10

15

20

The resulting plasmid, designated pLP3537-maf, thus differs from pLP3537 (Posno, 1991, supra) by the addition of two restriction sites. The 5' end of *xylA* was then subcloned into pLP3537-maf as follows: the PCR product *xylAB* was digested with *Sa*I and *Hind*III and the resulting 900 bp DNA fragment was purified by preparative agarose gel electrophoresis, ligated to pLP3537-maf previously digested with *Sa*I and *Hind*III and dephosphorylated with calf intestinal phosphatase, and transformed into *E. coli* DH5 α . The expected plasmid, designated pLP3537-*xylA*, was recovered from an ampicillin-resistant transformant. The remainder of *xylA* and *xylB* was then subcloned into pLP3537-*xylA* using a gel-purified 2.3 kb *Hind*III fragment obtained from digestion of pBS-*xylAB*. The ligation mixture was then transformed into *L. casei casei* 102S. The plasmid, designated p*xylAB*, was isolated from *L. casei casei* 102S and transformed into *Lactobacillus* MONT4⁺. Restriction analysis of plasmid DNA isolated from erythromycin-resistant colonies confirmed the presence of the expected plasmid in *Lactobacillus* MONT4⁺.

25

To determine whether removal of the regulatory gene, *xyIR*, had an effect on xylose assimilation in the presence of glucose, *Lactobacillus* MONT4⁺(p*xylAB*) and *Lactobacillus* MONT4⁺(pLP3537-*xyl*), grown in the presence of either glucose or xylose, were inoculated to an initial OD600 of 0.08-0.1 into MRS media containing 10 μ g/ml erythromycin and either 2% glucose, 2% xylose or 0.4% glucose + 1.6% xylose. The cultures were incubated at 37°C for up to 137 hrs. Growth, sugar utilization and product formation were monitored as described above.

Table 3
Lactate Yields after 137 hours

Strain	Media	Inoculum	Yp	Yp/s
Mont4+ (pLP3537-xyl)	2% glucose	glucose	91%	91%
Mont4+ (pxylAB)	2% glucose	glucose	93%	93%
Mont4+ (pLP3537-xyl)	2% xylose	xylose	76%	95%
Mont4+ (pxylAB)	2% xylose	xylose	82%	101%
Mont4+ (pLP3537-xyl)	0.4% glu+1.6%xyl	glucose	56%	103%
Mont4+ (pxylAB)	0.4% glu+1.6%xyl	glucose	74%	99%
Mont4+ (pLP3537-xyl)	0.4% glu+1.6%xyl	xylose	68%	102%
Mont4+ (pxylAB)	0.4% glu+1.6%xyl	xylose	80%	99%

The results presented in Table 3 demonstrate that improved lactate yields were obtained with MONT4+(pxylAB) based on consumed sugar (Yp/s) and total available sugars (Yp) in MRS media containing either 2% (w/v) glucose or 2% (w/v) xylose. In contrast to Mont4+ (pLP3537-xyl), no lag in growth or in lactate production was observed with Mont4+ (pxylAB) in MRS media containing 0.4% glucose and 1.6% xylose after the glucose was consumed in about 40 hrs. Both strains demonstrated the highest lactate yields Y_p in MRS media containing 2% (w/v) glucose, followed by 2% (w/v) xylose, 0.4% (w/v) glucose + 1.6% (w/v) xylose (xylose inoculum), and 0.4% (w/v) glucose + 1.6% (w/v) xylose (glucose inoculum). It is apparent from these results that the lactate yields based on total available sugars were greater with *Lactobacillus* MONT4+ (pxylAB), indicating that removal of *xylR* has improved xylose fermentation performance, either in the presence or absence of glucose.

Although the foregoing examples have been written with respect to specific embodiments, the spirit of the invention is not limited thereto and various modifications which would be obvious to one skilled in the art are intended to be encompassed herein as recited by the appended claims.

Claims

1. A microorganism of the genus *Lactobacillus* containing exogenous genes which is capable of fermenting xylose to lactic acid, wherein said microorganism without said genes is incapable of fermenting said xylose to lactic acid.
2. The microorganism of claim 1, wherein said microorganism is *Lactobacillus* MONT4.
3. The microorganism of claim 2, containing exogenous genes encoding xylose isomerase and xylulokinase.
4. The microorganism of claim 3, wherein said genes encoding xylose isomerase and xylulokinase are obtained from *Lactobacillus pentosus*.
5. The microorganism of claim 1, wherein said genes are located on a vector.
6. The microorganism of claim 5, wherein said vector is a plasmid.
7. A vector containing genes encoding xylose isomerase and xylulokinase obtained from *Lactobacillus pentosus*, but not containing the *xylR* gene.
8. The vector of claim 7, wherein the vector is a plasmid.
9. A process for producing lactic acid or lactate from lignocellulosic biomass using the microorganism of claim 3.
10. The process according to claim 9 wherein the lignocellulosic biomass contains xylose.
11. The process according to claim 9 wherein the lignocellulosic biomass contains xylose and glucose.
12. The process according to claim 9 wherein the lignocellulosic biomass contains xylose, glucose and arabinose.

AMENDED CLAIMS

[received by the International bureau on 17 February 1997 (17.02.97);
original claims 1-12 replaced by amended claims 1-10 (1 page)]

1. A homofermentative microorganism *Lactobacillus* MONT4 containing exogenous genes capable of homofermenting xylose to lactic acid, wherein said microorganism without said genes is incapable of fermenting said xylose to lactic acid.
3. The microorganism of claim 1, containing exogenous genes encoding xylose isomerase and xylulokinase.
4. The microorganism of claim 3, wherein said genes encoding xylose isomerase and xylulokinase are obtained from *Lactobacillus pentosus*.
5. The microorganism of claim 1, wherein said genes are located on a vector.
6. The microorganism of claim 5, wherein said vector is a plasmid.
7. A vector containing genes encoding xylose isomerase and xylulokinase obtained from *Lactobacillus pentosus*, but not containing the *xylR* gene in *Lactobacillus* MONT4.
8. The vector of claim 7, wherein the vector is a plasmid.
9. A process for producing lactic acid or lactate in high yields from lignocellulosic biomass by adding the microorganism of claim 3 into said lignocellulosic biomass to ferment sugars to lactic acid or lactate.
10. The process according to claim 9 wherein the lignocellulosic biomass contains xylose.

1/2

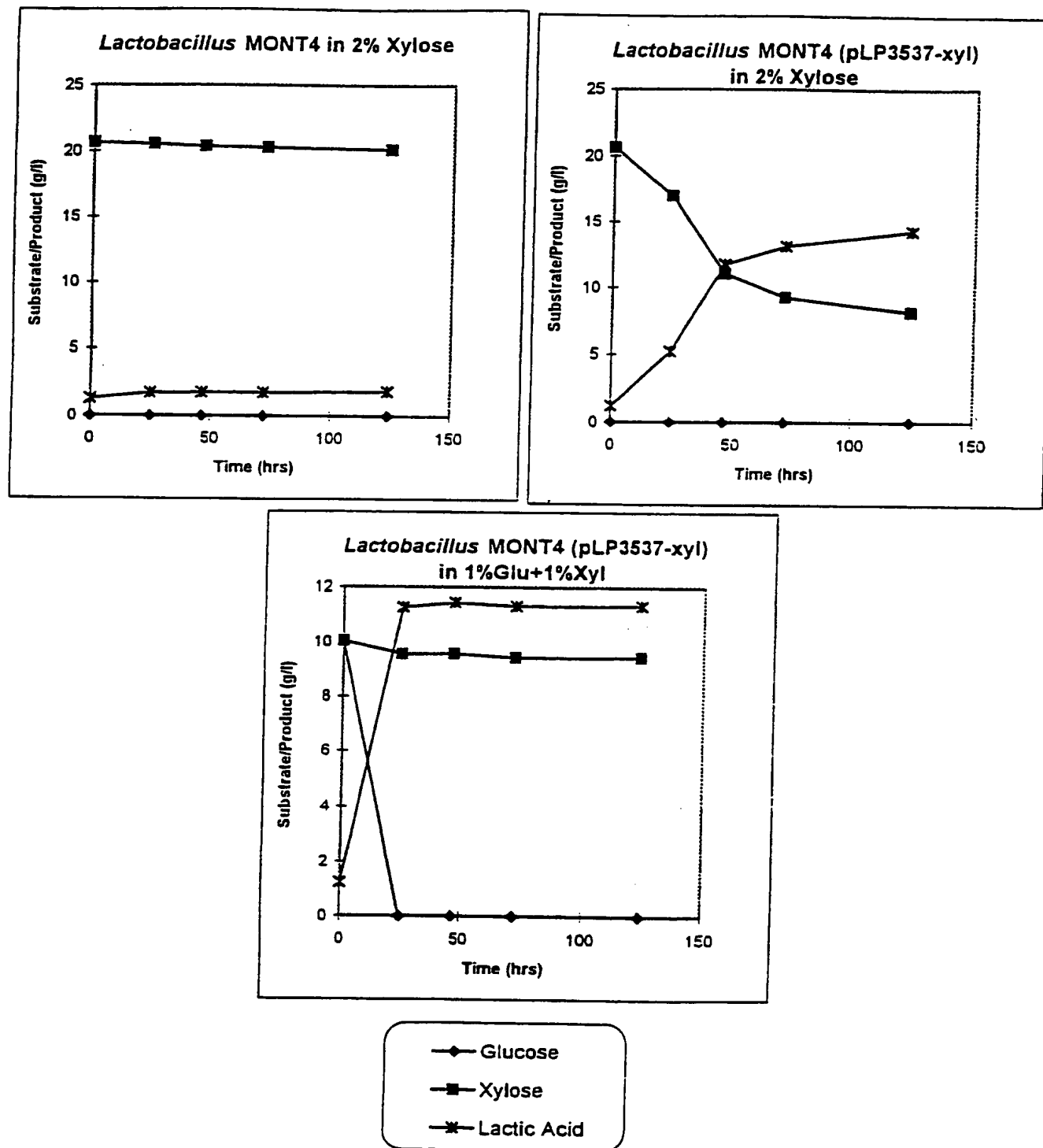


Fig. 1

2/2

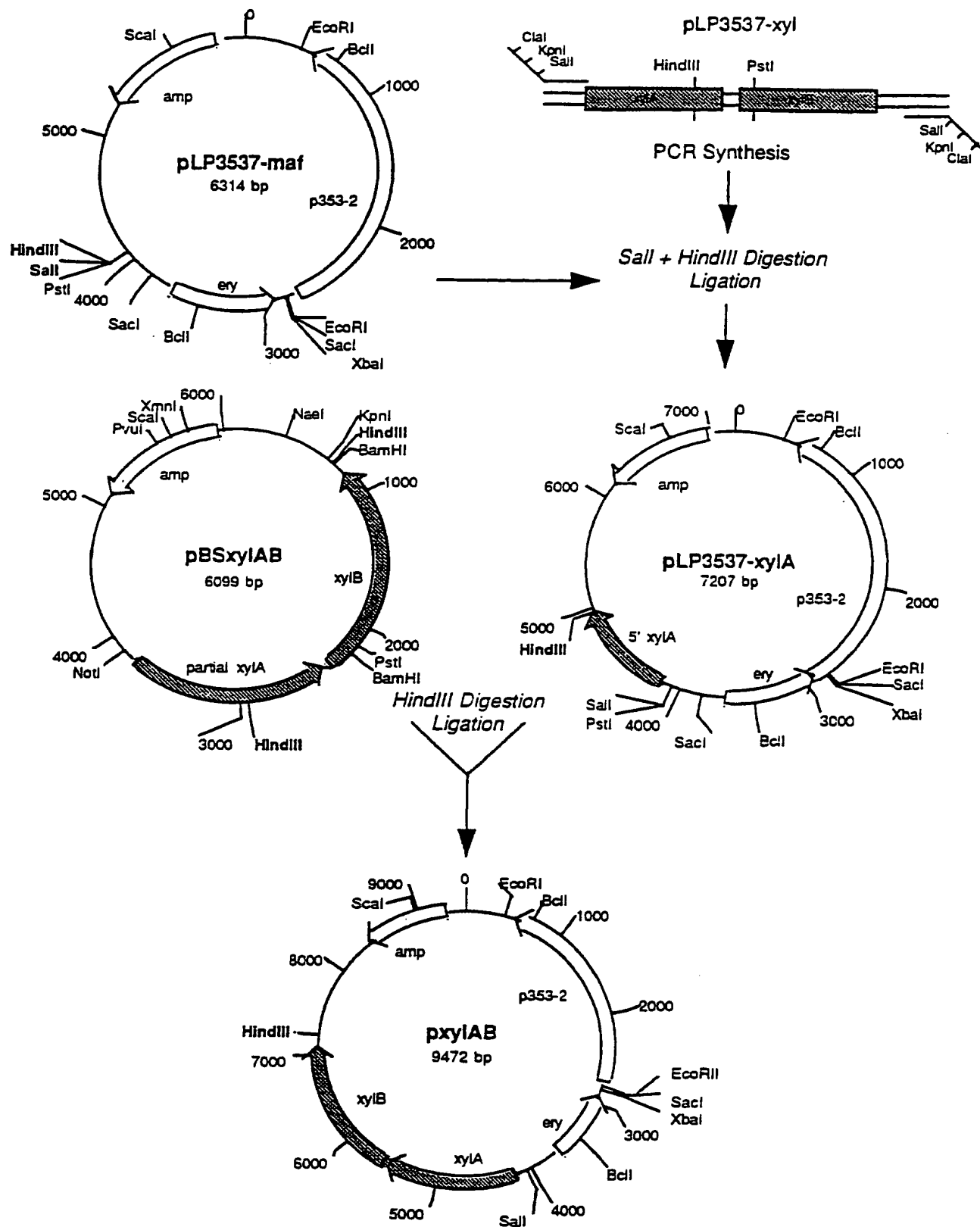


Fig. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/15442

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 1/20, 15/74; C12P 7/56

US CL : 435/139, 252.3, 252.9, 320.1,

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/139, 252.3, 252.9, 320.1,

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	Applied and Environmental Microbiology, Volume 57, No. 9, issued September 1991, M. Posno et al, "Complementation of the Inability of Lactobacillus Strains to Utilize D-Xylose with D-Xylose Catabolism-Encoding Genes of Lactobacillus pentosus", pages 2764-2766, especially abstract, and Figure 1.	1, 5, 6 ----- 2-4, 7-12
X ----- Y	Molecular and General Genetics, Volume 230, issued 1991, B.C. Lokman et al., "Organization and Characterization of Three Genes Involved in D-xylose Catabolism in Lactobacillus pentosus", pages 161-169, see especially page 162.	1, 5, 6 ----- 2-4, 7-12

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A document defining the general state of the art which is not considered to be of particular relevance	*X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E earlier document published on or after the international filing date	*Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*& document member of the same patent family
*O document referring to an oral disclosure, use, exhibition or other means	
*P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

30 DECEMBER 1996

Date of mailing of the international search report

17 JAN 1997

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

Nancy T. Vogel

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/US96/15442**C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Applied Bacteriology, Volume 44, issued 1978, P. Barre, "Identification of Thermobacteria and Homofermentative, Thermophilic, Pentose-utilizing Lactobacilli from High Temperature Fermenting Grape Musts", pages 125-129, see entire document.	2-4, 9-12
Y	Applied and Microbiology Biotechnology, Volume 24, issued 1991, K. Thompson et al., "Molecular Cloning in Lactobacillus helveticus by Plasmid pSA3::pVA797 Co-Integrate Formation and Conjugal Transfer", pages 334-338, see entire document.	2-4, 9-12
Y	Trends in Biotechnology, Volume 3, No. 11, issued November 1985, B. M. Chassy, "Prospects For Improving Economically Significant Lactobacillus Strains by 'Genetic Technology' ", pages 273-275, see entire document.	2-4, 9-12
Y	Journal of Bacteriology, Volume 171, No. 7, issued July 1989, P. Kreuzer et al., "Identification and Sequence Analysis of the Bacillus subtilis W23 xylR Gene and xyl Operator", pages 3840-3845, see entire document.	7, 8